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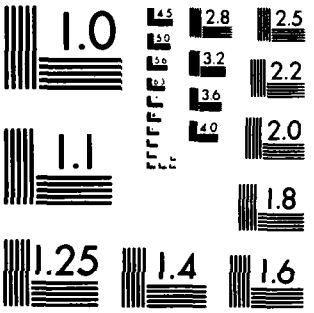

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AN AUTOMATED METHOD FOR DETERMINING ERYTHROCYTE OSMOTIC FRAGILITY

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NOTICES

This final report was submitted by personnel of the Clinical Pathology Branch, Clinical Sciences Division, USAF School of Aerospace Medicine, Aerospace Medical Division, AFSC, Brooks Air Force Base, Texas, under job order 7755-25-01.

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

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We have developed a new procedure for the dialysis method of determining erythrocyte fragility. Using a unique dialyzer cell (0.06 ml volume), this technic offers reduced cost, automation, multiple sample analysis, and micro volumes suitable for pediatrics and small animals. This technic also produces data suitable for data processing, storage, and retrieval and is more amenable to quality control formats.

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AN AUTOMATED METHOD FOR DETERMINING ERYTHROCYTE OSMOTIC FRAGILITY

INTRODUCTION

The increased sensitivity and reliability of laboratory instrumentation, together with better methods of quality control, have brought all clinical laboratory procedures under scrutiny. One of the tests for which current practices were questioned is the test tube, multiple salt concentration method of determining erythrocyte osmotic fragility (1).

The novel dialysis technic published by Danon (2) showed a promising approach to a more reliable and economic clinical procedure. We have designed a unique dialyzer cell and an automated technic that produces data suitable for computer processing and is more amenable to quality control formats than the test tube method. The small sample volume makes this technic appropriate for pediatric and small animal requirements.

MATERIALS AND METHODS

A. Materials:

1. Dialyzer cell (Fig. A-1). Details of construction and preparation appear in Appendix A.
2. Disposable, plastic, 3-ml cuvettes, 10x10x40 mm.
3. Dialysis membrane, tubular, 6 mm diameter.
4. Paraffin heating cup with embedding paraffin.
5. Micro-syringe with fixed needle, 250-1000 µl capacity.
6. Recording spectrophotometer, 500 nm.
7. Heparinized or acid-citrate-dextrose (ACD) blood collection tubes.
8. Barbital buffered salt solution. Details for preparation appear in Appendix B.

B. Procedure:

1. Make a dilution of 1 part blood to 9 parts buffer in a test tube sufficiently large for thorough aeration.
2. Place diluted blood on a rocking device for at least 1 hour.
3. Prepare dialyzer cells and store in buffer-filled cuvette at room temperature (see Appendix A).

4. Set up recording spectrophotometer at 500 nm with pinhole aperture. Set chart speed at 1 inch per minute. Set initial chart span at 2.5 optical density (O.D.) full scale. This span can be varied depending upon the O.D. of samples at maximum hemolysis.

5. Draw an aliquot of diluted blood into syringe and place syringe on rocker to keep cells suspended.

6. Momentarily remove dialyzer cell from buffer-cuvette and shake out buffer from inside the cell. Dry out the top of the cell with a cotton applicator and replace cell in the buffer-cuvette.

7. Immediately remove syringe from rocker, expel a few drops of sample to clear the needle, and load the dialyzer cell while it is sitting in the buffer-cuvette. (Insert needle in one access channel and completely fill the chamber from the bottom allowing air to escape through the second access channel.)

----------Stopping point for 2-3 minutes if needed----------

8. Fill test cuvette with distilled water and place in spectrophotometer.

9. At zero time remove loaded cell from buffer-cuvette, blot off excess buffer, place in distilled water-cuvette, and immediately start the recorder.

10. With no delay adjust slit to set the recorder pen at 100 (maximum scale) for zero time O.D.

11. Record diminishing O.D. of sample until stability is reached showing maximum hemolysis. (With cells in current use this total time is approximately 15 minutes.)

12. The pen position on the chart at the end of the run is taken as 100% hemolysis and that chart division recorded to the nearest 0.5 division.

13. The number of chart divisions between the beginning (100) and the ending division line is divided into intervals of 5% hemolysis (20 intervals) and marked on the chart. The time is measured, in seconds, for each percent interval from zero start to the point where the sample tracing crosses the calculated division line for that percent level.

RESULTS AND DISCUSSION

Examples of a run on a normal subject and a run using spherocytes are shown in Table 1. The first column of the table is the constant value, percent hemolysis. The first column of each subject data is the calculated chart division representing each percent hemolysis interval. The second column of the subject data is the elapsed time in seconds where the sample tracing crosses the calculated division line. This step in the method is illustrated on the mean curve of the normal range shown in Figure 1. In this form (time in seconds for each percent hemolysis interval), the data is easily stored in an array in a computer for patient files and for statistical analysis.

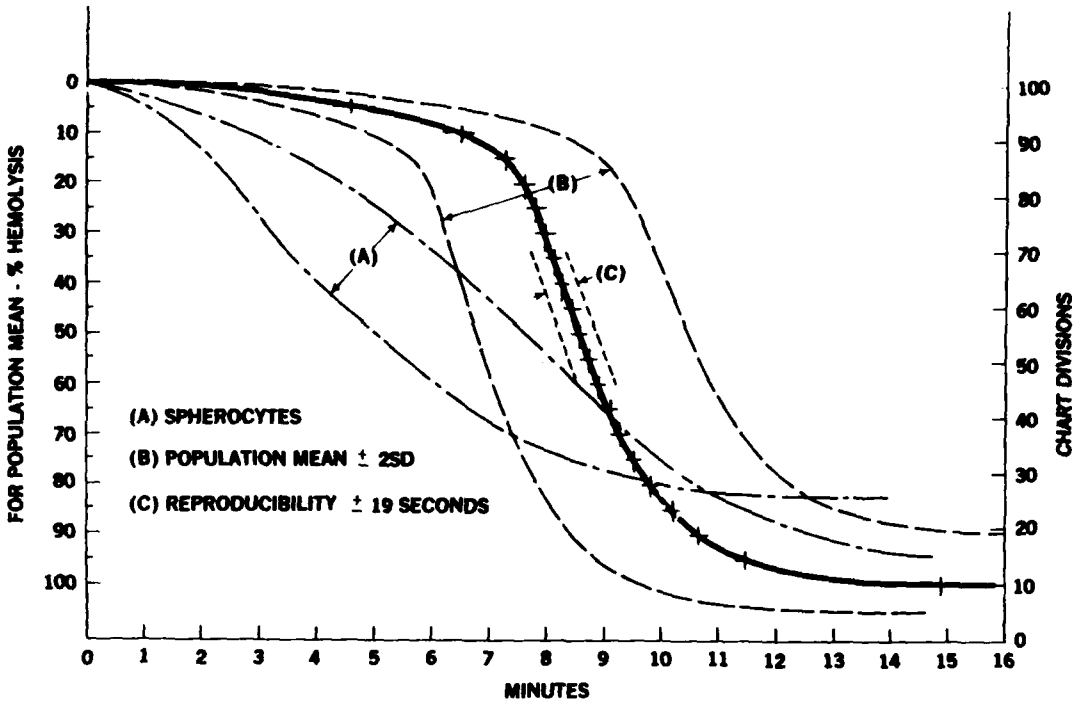

Figure 1. Normal and abnormal curves.

In Table 2 the mean and standard deviation (S.D.) are shown for our subject population at each percent interval from which a normal range curve can be derived (Fig. 1). To judge the reproducibility of the technic, we calculated the deviation from the mean as well as a coefficient of variation on replicate runs for each percent interval. For an overall picture of deviation we then took an average of the absolute values, as shown in Table 3.

The test tube technic used in most laboratories is performed by setting up tubes containing decreasing concentrations of salt solution, adding aliquots of blood, and calculating the percent hemolysis for each salt concentration (1). To compare the values obtained by dialysis with the test tube method, we found the approximate dialysis rate using an osmometer to measure the relative salt concentration in the cell at fixed time intervals. This dialysis data is shown in Table 4. We compared the two methods by interpolating between seconds of dialysis and salt concentration. An example of data comparing the two methods is shown in Table 5. Since the measured parameters of the two methods are different, direct comparison requires an interpolation between parameters that we feel negates the accuracy of statistical correlation.

The test tube method specifies a phosphate buffer whereas a barbital buffer is recommended for the dialysis technic. We examined both buffers with the test tube method and found similar rates of hemolysis at comparable ionic strength. While Danon (2) recommends heparin as the anticoagulant, we had need of ACD for other purposes and found that it worked equally well; therefore the test results reported here are on blood collected in ACD tubes.

TABLE 1. EXAMPLES OF TEST DATA

% Hemolysis	Normal Erythrocytes		Spherocytes	
	Chart Division[a]	Seconds[b]	Chart Division[a]	Seconds[b]
5	95.5	254	96.5	60
10	91.0	386	93.0	100
15	86.5	434	89.0	128
20	82.0	453	85.5	162
25	77.5	470	82.0	186
30	73.0	486	78.5	204
35	68.5	495	75.0	224
40	64.0	504	71.0	240
45	59.5	518	67.5	258
50	55.0	528	64.0	276
55	50.0	540	60.5	295
60	45.5	550	57.0	314
65	41.0	558	53.0	333
70	36.5	570	49.5	350
75	32.0	584	46.0	372
80	27.5	602	42.5	406
85	23.0	620	39.0	423
90	18.5	652	35.0	449
95	14.0	705	31.5	572
100	9.5	906	28.0	780

[a] Recorder chart division calculated for 5% intervals.

[b] Time in seconds, from 0 time to point where sample tracing crosses calculated division line.

TABLE 2. POPULATION NORMAL CURVE[a]

% Hemolysis	Mean Seconds	S.D.[b] Seconds
5	273	39
10	389	44
15	434	47
20	454	48
25	467	49
30	477	50
35	486	50
40	495	50
45	504	51
50	512	51
55	522	52
60	532	52
65	543	54
70	555	53
75	569	52
80	587	53
85	608	53
90	636	53
95	687	52
100	893	27

[a] Based on 90 samples.

[b] Standard deviation.

TABLE 3. REPRODUCIBILITY ON MULTIPLE RUNS

% Hemolysis	Average Deviation[a]	Average % C.V.[b]
5	13	6.5
10	15	5.7
15	17	5.9
20	17	5.6
25	17	5.6
30	18	5.6
35	18	5.6
40	18	5.6
45	19	5.6
50	19	5.5
55	19	5.6
60	19	5.6
65	20	5.5
70	21	5.6
75	22	5.7
80	21	5.2
85	20	5.1
90	19	4.5
95	19	3.9
100	22	3.1

[a]Average seconds deviation from the mean, 2-6 runs per sample.

[b]Average of the coefficient of variation on all samples.

TABLE 4. DIALYSIS RATE OF BUFFERED SALT SOLUTION

Minutes	% Salt
1	90
2	84
3	78
4	73
5	68
6	63
7	59
8	55
9	51
10	47
11	44
12	41
13	38
14	35
15	32

TABLE 5. COMPARISON OF TEST TUBE AND DIALYSIS METHODS

	Test Tube		Dialysis	
% Hemolysis	% Salt	Seconds[a]	% Salt[a]	Seconds
4	60	435	68	318
43	55	510	54	518
90	50	585	46	624
95	45	660	44	666
99	40	735	40	734
100	35	840	34	876

[a]Calculated from the dialysis rate.

One variable that contributes to the deviation in reproducibility of this technic is the quality of the dialysis membrane available. Wide differences in tubing diameter and pore size occur between manufacturers and smaller, but significant, differences occur throughout the length of a package of tubing from a single source. The small lengths of tubing used to prepare the cells make these differences very apparent on occasion. All routine values were used in the calculations of reproducibility reported here; therefore, an interested investigator may find, as we did, that some sections of tubing produce much better reproducibility than that shown in our tables.

We use a recording spectrophotometer equipped with a four-sample compartment and an automatic stepping function which we set at a 5-second interval. This instrument makes it practical to make at least two measurements on two patients simultaneously. A tracing of the normal range curves established in the laboratory can be overlaid on the chart of the day's run to quickly identify an abnormal patient.

To speed up calculation of chart divisions, a series of tables is made for each possible end-point from 0 chart division to an end-point of 30 in 0.5 division intervals. The absolute end-point is determined by the chart span and the hemoglobin content of the sample; therefore, the variability does not affect the test data. If a series of runs end below 0 or above 30, the chart span is adjusted to keep the curve in range.

The reproducibility of the dialysis method and the ease of determining abnormal fragility outside the normal range, together with the type of numeri data produced, the advantage of automated analysis, and the reduced time and cost, make this dialysis technic appear most favorable compared to the test tu method. The small sample size also makes this procedure advantageous for pediatric work and small animal requirements. Although this method proposes a different reporting procedure, the precision presents a more definitive picture which may prove a better diagnostic tool for the physician.

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APPENDIX A

PREPARATION OF DIALYZER CELL

The dialyzer cell (Fig. A-1) is constructed of plastic in two pieces. The blade is cemented into the top. The final sizing and polishing of the blade depends upon the diameter of the membrane used. Although manufacturers state a diameter of 6 mm (usually specified as 10 mm-flat width), there are differences in size between commercial sources.

Preparation of cells:

1. Cut membrane to length, measuring from the top of the blade to 3 mm below the window.

2. Soak the membrane until it is soft and slip it over the blade snug to the top.

3. Blot the outside and dip the lower tip of the blade in melted paraffin up to 1 mm below the window, thus sealing the bottom of the tubing.

4. After the paraffined tip has cooled, place the cell in a cuvette filled with buffer solution.

These units can be prepared in advance and stored in buffer at room temperature until needed.

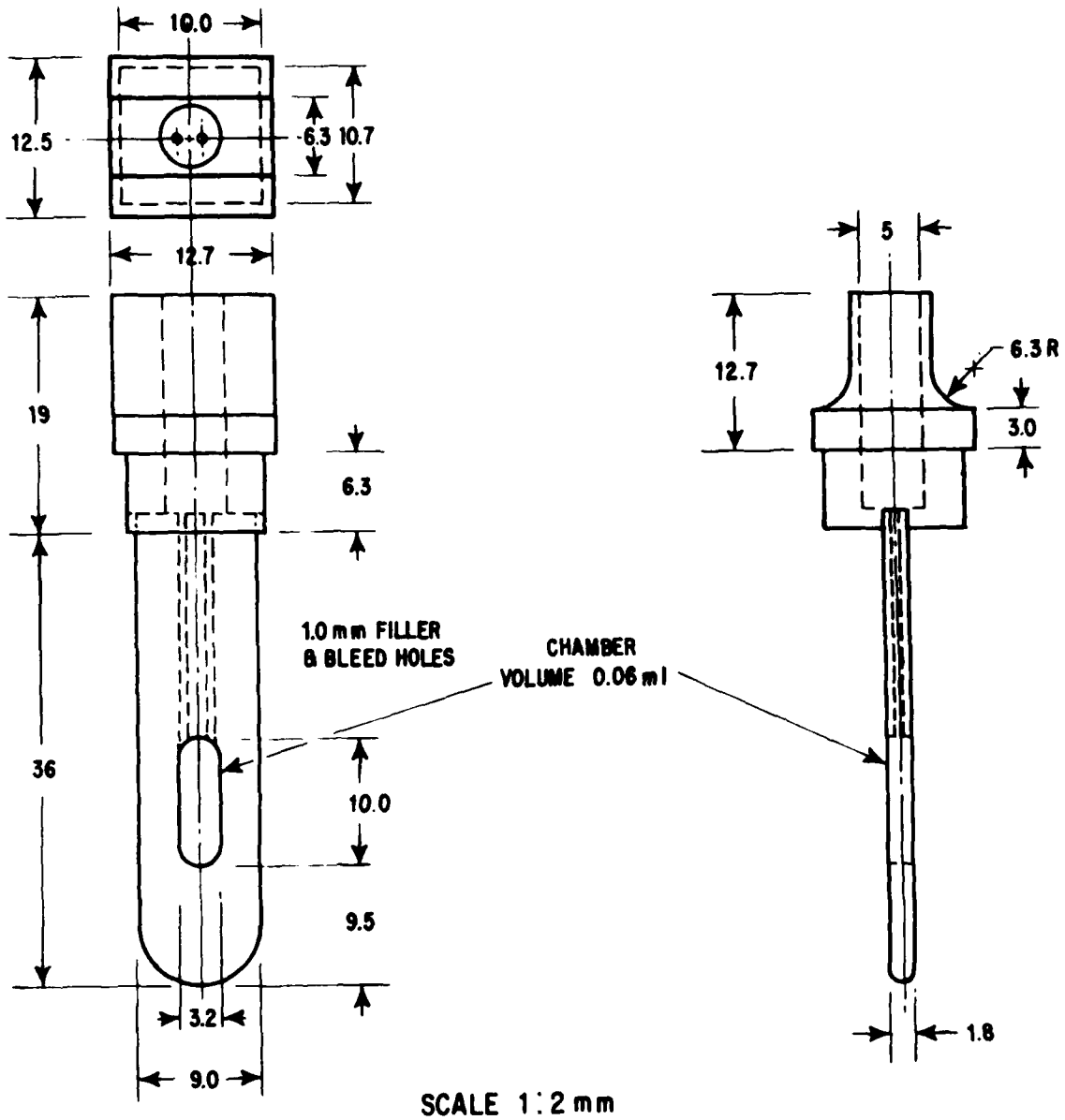
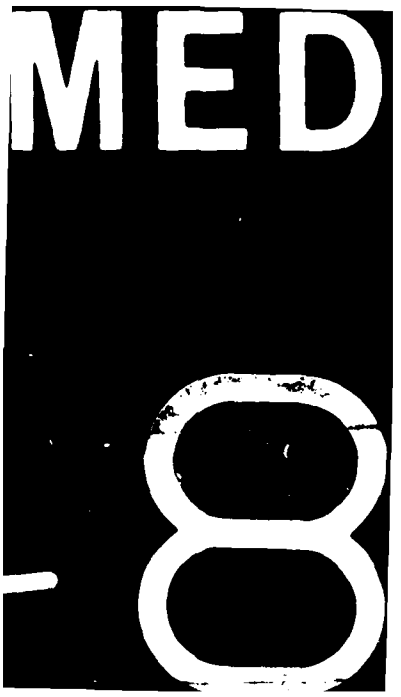


Figure A-1. Specifications for dialyzer cell.

APPENDIX B

PREPARATION OF BUFFER

1. Place 8.5g of sodium chloride in a 1-liter volumetric flask. Add 900 ml of distilled water.
2. Add 24 ml of 0.1M sodium barbital solution.
3. Add 16 ml of 0.1M hydrochloric acid solution.
4. Adjust the pH to 7.4 using sodium hydroxide or hydrochloric acid.
5. Adjust the volume to 1 liter.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